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New sources of high-temperature tolerant rhizobia for *Phaseolus vulgaris* L.

MARIANGELA HUNGRIA[1], AVÍLIO ANTÔNIO FRANCO[2] and JANET I. SPRENT[3]
[1]*EMBRAPA-CNPSo, Caixa Postal 1061, CEP 86001-970, Londrina, PR, Brazil,* [2]*EMBRAPA-CNPBS, Caixa Postal 74505, CEP 23851, Seropédica, R J, Brazil and* [3]*University of Dundee, Department of Biological Sciences, Dundee, DD14HN, Scotland*

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Abstract

Common bean (*Phaseolus vulgaris* L.) represents an important crop in tropics, but previous screenings of *Rhizobium leguminosarum* bv. *phaseoli* did not show strains that could fix N_2 in symbiosis with bean at temperatures higher than 35°C (Hungria and Franco, 1993). However, there are other rhizobia and bradyrhizobia species that nodulate some tropical leguminous trees and can fix N_2 at high temperatures. In a trial of rhizobial strains isolated from leguminous trees, we found that 14 out of 21 isolates from Gliricidia, Lonchocarpus and Leucaena were also able to nodulate common beans at optimal temperatures (28/23°C, day/night). When we exposed beans inoculated with these strains to high temperature conditions, 40°C/8 h/day, some of them accumulated at flowering time as much or more N as bean plants receiving mineral N. These broad host-range sources of rhizobia capable of fixing nitrogen with bean at high temperature seem to have the potential to improve yields in tropical soils.

Introduction

Nodulated legume species vary in their tolerance to high temperature. The common bean (*Phaseolus vulgaris* L.) is generally considered to be very sensitive (Piha and Munns, 1987), although it is an important crop in tropical countries. Rhizobium strains also vary in heat tolerance but in a previous work, the screening of strains of *R. leguminosarum* bv. *phaseoli* in Brazil did not yield any that could fix N_2 at 35 and 38°C (Hungria and Franco, 1993). Also, two thermal shocks at flowering time, each of 8 h at 40°C, decreased nitrogenase activity by 70%, and plants started to recover only after one week, due to the formation of new nodules (Hungria and Franco, 1993). In contrast, non-inoculated beans receiving mineral N were not affected by this short heat shock treatment.

Genes responsible for nodulation and N_2 fixation in *R. leguminosarum* bv. *phaseoli* are localized on a single replicon, the symbiotic (Sym) plasmid. The genome is complex, containing many reiterated DNA sequences that might provide sites for recombination and genomic rearrangements (Flores et al., 1987; Martinez et al., 1985). With temperature increases, plasmid deletions (Trevors, 1986) and genomic rearrangements (Soberón-Chávez et al., 1986) may happen, resulting in loss of symbiotic properties.

Some nodulated legume trees are less sensitive than common bean to high temperatures. Cunha and Franco (1988) studied ten species of leguminous trees and observed that *Leucaena leucocephala* and *Prosopis juliflora* inoculated with some heat-tolerant strains retained nitrogenase activity not only at 35 but also at 38°C.

Many legumes are specific in their rhizobial

requirements (Nutman, 1981; Vincent, 1980). However, there are records of soybean being nodulated and fixing N_2 with both *Bradyrhizobium japonicum* and *R. fredii* (Keyser et al., 1982). Also, *Lotus pedunculatus* Cav. is nodulated by *R. Ioti* and *Bradyrhizobium* sp. (Pankhurst and Layzell, 1984), and there are similar results for *Cajanus cajan* L., *Cicer arietinum* L. (Bromfield et al., 1983) and *Vigna unguiculata* (L.) Walp. (Dakora and Vincent, 1984). Another bacterium, *Rhizobium* sp. NGR234 nodulates a wide-range of legumes, some of them evolutionarily divergent (Stanley and Cervantes, 1991). More recently, several strains able to nodulate beans were classified in the new species *Rhizobium tropici*, and characterized by being also able to nodulate *Leucaena esculenta* and *Leucaena leucocephala* (Martinez-Romero et al., 1991).

Since in previous experiments we were not able to select *R. leguminosarum* bv. *phaseoli* strains that could fix N_2 at high temperatures, we describe here our attempts to select rhizobial strains isolated from leguminous trees for the ability to nodulate and fix N_2 with common bean at high temperatures.

Materials and methods

Three experiments were performed under greenhouse conditions at EMBRAPA-CNPBS, km 47, Rio de Janeiro, Brazil.

The first tested the nodulation of common beans (*Phaseolus vulgaris* L.) with 76 rhizobial strains: 69 isolates from 18 leguminous tree species, fours trains of *R. fredii* (or *Sinorhizobium fredii*, Chen *et al.*, 1988) two strains of *R. tropici*, CIAT 899 and CFN 299 (Martinez-Romero et al., 1991) and one strain of *R. leguminosarum* bv. *phaseoli*, CNPAF 146. Strains were grown on YM medium (Vincent, 1970) for five days at 28°C, and were then inoculated on cv. Negro Argel. Alkali or acid production in the culture medium was observed by the addition of bromothymol blue (5 mg L^{-1} of a 0.5% solution). Seeds were surface-sterilized (Vincent, 1970) and soaked for 1 h in 1 mL of inoculum (about 10^8 cells mL^{-1}) for each 15 seeds. Four seeds were planted per sterilized Leonard jar (Vincent, 1970) previously filled with sand and vermiculite (1/2, v/v). Four days after emergence (DAE) plants were thinned to two per jar.

The experiment was laid out in a completely randomized block design with five replicates. Plants were grown at 28/23°C, day/night, and received N-free nutrient solution weekly as described previously (Hungria and Franco, 1993). Plants were harvested at the beginning of flowering (28 DAE) and were evaluated for nitrogenase activity, nodule dry weight, shoot dry weight and total N as described before (Hungria and Franco, 1993).

The second experiment was designed to test the heat tolerance of beans cv. Negro Argel inoculated with nine strains isolated from leguminous trees (selected from the first experiment) and three strains isolated from beans. A non-inoculated treatment received 50 mg N/plant/week (as KNO_3), giving a total of 200 mg of N.

Plants were grown in pots placed into tanks of circulating water as described before (Hungria and Franco, 1993) with thermostats controlling the temperature. For the high temperature treatment (40°C/8 h/day), heating was turned on from 7:00 a.m. to 5:00 p.m., taking about 2 h to reach the desired temperature. After thermostats were switched off, the temperature of the pots quickly decreased to 23°C. Air temperatures around shoots averaged 29/23°C, day/night.

Water and N-free nutrient solution were given as described before (Hungria and Franco, 1993). Plants were harvested at the beginning of flowering, 30 DAE, and nodule number and total N in shoots were determined (Hungria and Franco, 1993).

The third experiment was designed to test the heat tolerance of three legumes: *Phaseolus vulgaris*, cultivar Negro Argel, *Leucaena leucocephala* and *Prosopis juliflora* (seeds produced at EMBRAPA-CNPBS) with five of the rhizobial strains used in experiments 1 and 2 and two strains that fixed N_2 with Prosopis at high temperatures (Cunha and Franco 1988) (Table 1). For *P. vulgaris*, a non-inoculated control receiving 50 mg N (as KNO_3)/plant/week was also included. Plants were grown in the tank system described before and with root tempera-

Table 1. Leguminous species and *Rhizobium* strains used in experiment 3

Test host species	Strain	Host of isolation
Prosopis juliflora	Br 4002, Br 4007	*Prosopis juliflora*
	Br 814, Br 816, Br 817	*Leucaena leucocephala*
Leucaena leucocephala	Br 814, Br 816, Br 817	*Leucaena leucocephala*
	CIAT 899, CFN 299	*Phaseolus vulgaris*
Phaseolus vulgaris	CIAT 899, CFN 299	*Phaseolus vulgaris*
	Br 814, Br 816, Br 817	*Leucaena leucocephala*

tures of 28, 35 or 39°C/8 h/day and 23°C at night. Phaseolus and Leucaena were harvested at 30 DAE and Prosopis was harvested at 60 DAE. Parameters evaluated were: acetylene reduction activity and relative efficiency, as described before (Hungria and Franco, 1993). Total nitrate reductase, dry weight and total N were evaluated only for *P. vulgaris*, also as described before (Hungria and Franco, 1993).

The second and third experiments were laid out in a split-plot design with four replicates, and mean values were statistically compared using the Tukey's test.

Results

Fourteen strains isolated from three of the tree species (Gliricidia, Leucaena and Lonchocarpus) and two strains of *S. fredii* were also able to form effective nodules on beans (Table 2). Strain effectiveness on common bean did not correlate with the origin of the legume subfamily: Gliricidia and Lonchocarpus belong to the Papilionoideae, and Leucaena belongs to the subfamily Mimosoideae. Strains that did not nodulate beans are listed in the footnote on Table 2. As there were not significant differences

Table 2. Nodulation and N_2 fixation in common bean (*Phaseolus vulgaris* L.) inoculated with *Rhizobium* strains isolated from bean, soybean and leguminous trees. Numbers represent the mean for all strains that nodulated bean. Plants harvested at 28 DAE with five replicates per strain

Host species	Number of strains tested	Alkali/acid production in YM medium	Nodule dry weight (mg/plant)	Nitrogenase activity (μmol C_2H_4/plant/h)	Shoot	
					Dry weight (g/plant)	Total N (mg N/plant)
	Leguminous trees					
Gliricidia spp.[z]	6 (6)[y]	Acid	310 c	31.4 c	2.3 ab	71.3 bc
Leucaena spp.	12 (5)	Acid	510 a	52.5 ab	2.2 ab	60.6 cd
Lonchocharpus spp.	3 (3)	Alkali	280 c	26.2 c	1.9 b	49.7 d
	Other grain legumes					
Glycine max (*S. fredii*)	4 (2)	Acid	340 bc	45.5 b	1.6 b	63.2 cd
	R. tropici					
P. vulgaris	CIAT 899	Acid	380 abc	54.0 ab	2.6 ab	85.5 b
	CFN 299	Acid	295 c	48.5 ab	1.7 b	47.3 d
	R.l. bv. *phaseoli*					
P. vulgaris	CNPAF 146	Acid	480 ab	60.1 a	2.9 a	110.0 a

[z] Subfamilies: Papilionoideae (Phaseolus, Glycine, Gliricidia, Lonchocarpus); Mimosoideae (Leucaena).

[y] Number of strains tested and, between parentheses, number of strains that nodulate beans.

[x] Number of *Rhizobium* strains tested that failed to nodulate bean: subfamily Mimosoideae – *Acacia* spp. (3), *Mimosa* spp. (3), *Prosopis* spp. (3), *Enterolobium* spp. (6), *Piptadenia* spp. (3), *Albizia* spp. (3), *Pithecellobium* spp. (3), *Parapiptadenia* spp. (3); Subfamily Caesalpinioideae – *Dimorphandra* spp. (3); Subfamily Papilionoideae – *Sesbania* spp. (3), *Clitoria* spp. (6), *Poecilanthe* spp. (3), *Dalbergia* spp. (3), *Bowdichia* spp. (3).

between strains isolated from the same host, means for all strains are given.

Effectiveness of the nodules was confirmed by the high levels of acetylene reduction activity and total N accumulated in shoots of plants inoculated with strains from Gliricidia, Leucaena and soybean (Table 2). All these strains were fast growers and produced acid in culture medium. Strains from Lonchocarpus, characterized by slow growth and alkali production, accumulated as much N as CFN 299, also a *Rhizobium tropici* strain with high efficiency of N_2 fixation.

In the second experiment, at high temperatures (40°C/8 h/day), plants inoculated with four tree strains (three strains, Br 814, Br 816 and Br 817, isolated from Leucaena and one, Br 6010, from Lonchocarpus) accumulated at least as much N as plants receiving 200 mg N until 30 DAE (50 mg N/week) (Table 3). Total N was also not statistically different between plants receiving mineral N and plants inoculated with Br 8802 (from Gliricidia) and Br 6009 and 6011 (from Lonchocarpus).

R. tropici CIAT 899 effectively fixed N_2 at high temperature with common bean. Although *R. leguminosarum* bv. *phaseoli* CNPAF 146, specific for common bean, was the best strain at 28°C (Table 2), it nodulated poorly and accumulated little nitrogen at 40°C (12 mg/plant, of which 7.5 mg N came from the seed) (Table 3).

In the third experiment, using different host species, when temperatures were elevated to 35 and 39°C, nitrogenase activities and relative efficiencies apparently decreased for all species and strains used (Table 4). There were no significant differences between strains grouped in each treatment, so the values were averaged over the strains. Rates of acetylene reduction were lower for Prosopis and Leucaena, but the initial growth of these tree species is slower than for common bean. From 28 to 35°C, acetylene reduction was decreased by an average of 32 and 49%, respectively for Prosopis and Leucaena, and of 66% for common beans. When the temperature increased from 35 to 39°C the decrease was on average of 57 and 72%, for Prosopis and Leucaena, and of 24% for beans (Table 4). Shoot nitrate reductase of common bean receiving 50 mg N/week was also affected

Table 3. Effect of *Rhizobium* strain on nodule number and N accumulated in shoots of *Phaseolus vulgaris* grown at high temperatures (40°C/8 h/day). Mean of 4 replicates of plants harvested at 30 DAE

Rhizobium isolated from	*Rhizobium* strain	Number of nodules (no/plant)	Total N (mg N/plant)
Gliricidia	Br 8801	8 e[z]	15 d
	Br 8802	20 cd	22 cd
	Br 8803	12 e	14 d
Leucaena	Br 814	46 b	41 ab
	Br 816	40 bc	41 ab
	Br 817	68 a	52 a
Lonchocarpus	Br 6009	14 de	28 c
	Br 6010	60 ab	48 ab
	Br 6011	20 cde	22 cd
P. vulgaris (*R. tropici*)	CIAT 899	67 a	50 a
	CFN 299	24 cde	30 c
(*R. leguminosarum* bv. *phaseoli*)	CNPAF 146	2 e	12 d
Uninoculated control (200 mg N)		zero	35 bc

[z] Values within columns followed by the same letter are not significantly different (Tukey's test, $p = 0.05$).

Table 4. Acetylene reduction activity and relative efficiency (RE) of three leguminous species grown at 28, 35 and 39°C. Prosopis was harvested at 40 DAE and Leucaena and Phaseolus were harvested at 30 DAE. Mean of 4 replicates

Legume species/ *Rhizobium* strain	Acetylene reduction activity (μmol C_2H_4/plant/h)			Relative efficiency		
	28°C	35°C	39°C	28°C	35°C	39°C
Prosopis/Br 4002, Br 4007	1.02 a[z]	0.60 b	0.30 b	0.80 a	0.79 a	0.65 b
Prosopis/Br 814, Br 816, Br 817	0.80 a	0.62 a	0.22 b	0.84 a	0.72 b	0.68 c
Leucaena/Br 814, Br 816, Br 817	3.20 a	1.51 b	0.40 c	0.86 a	0.75 b	0.70 b
Leucaena/CIAT 899, CFN 299	2.10 a	1.15 b	0.36 c	0.85 a	0.70 b	0.71 b
Phaseolus/Br 814, Br 816, Br 817	17.27 a	8.02 b	5.30 b	0.91 a	0.61 b	0.60 b
Phaseolus/CIAT 899, CFN 299	14.30 a	3.14 b	2.70 b	0.91 a	0.50 b	0.49 b

[z] There were no significant differences between strains grouped in one line. Values given are thus averaged over the strains, and in each line, means followed by the same letter are not different for acetylene reduction activity and relative efficiency rates (Tukey's test, $p = 0.05$).

Table 5. Shoot nitrate reductase of *Phaseolus vulgaris* grown at three different temperatures. Plants were harvested at 30 DAE and received 200 mg N/plant. Mean of 4 replicates

medium	Nitrate reductase activity (μmoles NO_2^-/g fresh weight/h)		
	28°C	35°C	39°C
$-NO_3^-$	0.48 a[z]	0.22 b	0.22 b
$+NO_3^-$	2.12 a	1.63 b	0.97 c

[z] Values followed by the same letter are not statistically different ($p = 0.05$, Tukey's test).

by high temperatures, decreasing 23% when plants received daily a temperature treatment of 35°C/8 h and with a further decrease of 40% when the temperature increased to 39°C (Table 5). When beans were inoculated with strains isolated in Brazil from Leucaena trees, Br 814, Br 816 and Br 817, symbiosis was more heat-tolerant than with *Rhizobium tropici* strains CIAT 899 and CFN 299. Either under optimal temperature conditions (28°C) or at 35 and 39°C, beans inoculated with strains isolated from Leucaena or with *R. tropici* strains accumulated as much N as plants receiving mineral N (Fig. 1).

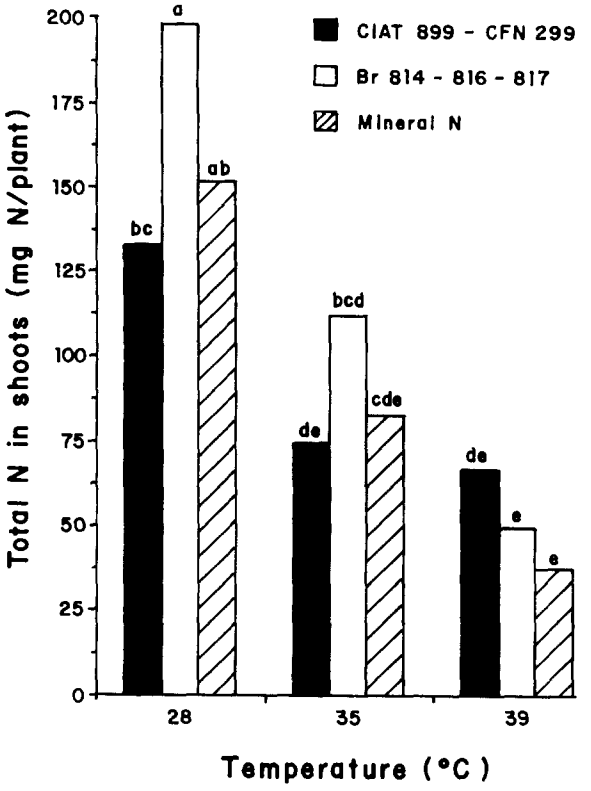

Fig. 1. Total N accumulated in shoots of *Phaseolus vulgaris* nodulated or receiving 50 mg N/plant/week and grown at 28, 35 and 39°C. Mean of 4 replicates of each strain combination harvested at 30 DAE. Values followed by the same letter are not significantly different (Tukey's test, $p = 0.01$).

Discussion

In a previous work, we failed to obtain *R. leguminosarum* bv. *phaseoli* strains that could nodulate and fix N_2 in plants grown at 35 and 38°C, and one thermal shock, of 40°C/8 h/day on plants growing at 28°C decreased nitrogenase activity by 70% (Hungria and Franco, 1993). In the tropics, soil temperatures between 40 and 60°C are not uncommon (Munévar and Wollum, 1981) and may explain the failure of N_2 fixation in field-grown common bean. However, some

rhizobial strains from tree legumes can nodulate and fix N_2 in temperatures as high as 40°C (Cunha and Franco, 1988), and could represent a genetic source for nodulation at high temperatures with other species.

We showed in the first experiment that some rhizobial strains isolated from Leucaena, Gliricidia and Lonchocarpus form effective nodules with common beans. Almost 30 years ago, Lange (1961) reported nodulation of beans by strains from other tropical legumes, although he did not mention whether the nodules were effective. Later, Martínez et al. (1985) also showed that beans could be nodulated and fix N_2 with rhizobial strains from other species. First, Martínez et al. (1985) proposed that strains able to nodulate both beans and Leucaena should be classified as a symbiotype II, characterized also by having a single copy of the *nif* genes, a lower level of genomic rearrangements and consequently, lower potential loss of symbiotic properties (Martínez et al., 1988).

More recently, a new species, *Rhizobium tropici*, was proposed for the symbiotype II strains (Martinez-Romero et al., 1991), characterized also by the ability of cultures to growth under high temperatures (40°C). However, previous reports have shown that the ability of rhizobia and bradyrhizobia to grow in culture medium at high temperatures (up to 47°C) does not necessarily relate to the N_2 fixation under temperature-stress conditions (Karanja and Wood, 1988; La Favre and Eaglesham, 1986). Consequently, our results show that some rhizobial strains, isolated from tropical tree legumes and able to nodulate beans, were also able to maintain N_2 fixation even at extremely high temperature conditions (40°C/8 h/day). In the same experiment, our best strain at optimal temperature conditions, *R. leguminosarum* bv. *phaseoli* strain CNPAF 146, did not fix N_2 at high temperatures with common beans.

We conclude that N_2 fixation with beans at high temperatures can be successful using strains with a broad host-range, capable of nodulating both tropical legume trees and common bean. The use of these elite strains in inoculants may improve bean yields in tropical soils subjected to high temperatures. Further investigations into the genetics and physiology of these high-temperature tolerant symbioses are in progress.

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